5-1972

Spermiogenesis in Two Male-Sterile Third-Chromosome Mutants of Drosophila melanogaster

Richard F. Wilkinson
Utah State University

SPERMIOGENESIS IN TWO MALE-STERILE THIRD-CHROMOSOME MUTANTS OF *DROSOPHILA MELANOGASTER*

by

Richard F. Wilkinson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

ZOOLOGY

Approved:

_____
Major Professor

_____
Committee Member

_____
Committee Member

_____
Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

1972

ACKNOWLEDGEMENTS

I am especially thankful to my major professor, Hugh P. Stanley, for his help and encouragement during this study. I also thank James T. Bowman for his valuable advice and also for his assistance with *Drosophila* procedures. Thanks are also extended to Rex S. Spendlove for his professional assistance.

I also give thanks to my wife, Judy, and to my children, Mike and Mark, for their patience and understanding.

Richard F. Wilkinson

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

ABSTRACT

Spermiogenesis in two male-sterile Third-Chromosome mutants of *Drosophila melanogaster*

by

Richard F. Wilkinson, Master of Science

Utah State University, 1972

Major Professor: Hugh P. Stanley
Department: Zoology

An ultrastructural study of sperm differentiation was made on two male-sterile mutants of *Drosophila melanogaster*. The mutations were induced on the third chromosome with the monoalkylating agent ethyl methanesulfonate and were maintained in stock cultures over a balancer chromosome using a system of multiple inversions. The stages of sperm differentiation, a most dramatic form of cellular differentiation, were then studied at the electron microscope level to determine the effect of the mutations on sperm structure and to correlate those changes with normal sperm development.

Studies of the genetic control of cellular differentiation have been hampered by difficulties in devising an *in vitro* system. *Drosophila melanogaster* provides an excellent system for these types of studies since the genome is well characterized, sperm development is a continual process, it is a higher organism with many complex interactions and normal sperm differentiation has been well characterized and can serve as the control over such genetic manipulations.

Analysis of the first of the two mutants studied, ms(3)10R, presents some evidence for an inductive action of cytoplasmic microtubules on the condensation of chromatin in the nuclei of developing sperm which, in turn, influences the normal differentiation and shaping of the sperm head.

Additionally, the study of this mutant appears to indicate that this locus exercises an organizational rather than a structural control.

The second mutant studied, ms(3)3R, shows normal sperm differentiation until a very late stage of development. The sperm tails then proceed to degenerate in an ordered fashion that differs from the degenerative changes to the sperm tails described for Y-chromosome mutations and deficiencies. In addition to the ultrastructural changes seen, the male-sterile flies fail to copulate. It appears, therefore, that this genetic locus on the third chromosome functions in the maintenance of sperm structure and also the normal behavior of the flies. The behavioral abnormality observed suggests that the mutation affects the normal endocrine system of Drosophila melanogaster.

(44 pages)

INTRODUCTION

It is obvious that cytodifferentiation must be under control of the cell genome, but the study of genetic control of cell differentiation has been given only limited attention. One experimental system that lends itself to such a study is spermatogenesis in Drosophila melanogaster. This is an excellent system for the study of cell differentiation because it is a continual process throughout the life span of the male fly, it has well defined initial and terminal points, and there is a dramatic change in cellular morphology throughout the process of spermatogenesis.

Previous approaches to the genetic control of spermatogenesis in Drosophila melanogaster has been largely restricted to mutations or deletions of the Y chromosome. Hess and Meyer (1968) reviewed the activity of the Y chromosome in spermatogenesis and provided convincing evidence that mutations on the Y chromosome result in the disorientation of elements of the spermatid rather than the lack of synthesis that leads to structural elements. Similarly, Kiefer (1966, 1968, 1969, 1970) studied the effects of Y-chromosome mutations and deletions on spermiogenesis and concluded that the Y chromosome serves an organizational rather than structural role. These conclusions suggest, therefore, that the structural proteins and enzymes must be coded on the X and/or autosomal chromosomes. Autosome mutations resulting only in male sterility were induced on chromosome two by Edmonton (see Lindsley and Grell, 1967) but were subsequently lost. Therefore, successful isolation of male sterile flies due to autosome mutation was conducted in this laboratory. Spermiogenesis in two of these mutations on chromosome two were studied at the electron microscope level (Romrell, Stanley and Bowman, 1972a,b).

A detailed study on normal spermatogenesis in Canton-S wild type was recently published (Stanley, Bowman, Romrell, Reed and Wilkinson, 1972) and served, along with the work on spermatogenesis by Tates (1971), as controls to which comparisons of change in the male sterile mutant testes were made.

The ultrastructural characterization of spermiogenesis in two male-sterile third-chromosome mutants of Drosophila melanogaster provides the basis for this study.

REVIEW OF LITERATURE

Normal spermatogenesis in Drosophila

The testes of Drosophila can be arbitrarily divided into four regions that delineate areas in which specific spermatogenic events occur. The apical end is that portion of the testis found most distal to the external genitalia of the male fly. Within this region are found apical cells and predefinitive spermatogonia. The cells of the apical region have been reported to serve an androgenic function in insects (Naisse, 1966a). The predefinitive spermatogonia undergo mitotic divisions that represent the initial stages of spermatogenesis in Drosophila. Of the two cells that are derived from the predefinitive spermatogonium, one becomes a definitive spermatogonium that will develop into the spermatozoa. The other division product remains as a predefinitive spermatogonium, therefore assuring a constant supply of cells for spermatogenesis. The definitive spermatogonium mitotically divides four times to give 16 primary spermatocytes. The meiotic divisions that follow will result in 64 haploid spermatids which subsequently differentiate into the mature spermatozoa (Cooper, 1950; Tates, 1971). These differentiative processes are found in the coiled mid-region of the testis. Testicular ducts, a constriction at the basal region of the testis, and seminal vesicles comprise the remaining portions of the testes. The mature spermatozoa pass through the testicular duct and into the seminal vesicle where they are stored (Cooper, 1950).

The primary spermatocytes are phenotypically characterized by large, pleomorphic nuclei and large nucleoli. The mitochondria are randomly dispersed throughout the relatively sparce cytoplasm. The meiotic divisions, which occur in rapid succession, give spermatids with nearly circular

nuclei, each with a small homogenous nucleolus. The mitochondria in the spermatid cytoplasm fuse into a large syncytial mass referred to as the Nebenkern, and then divide into two Nebenkern derivatives, one of which forms a paracrystalline structure (Tates, 1971). Axonemal development follows the fairly typical insect pattern that results in a "9+9+2" microtubular pattern.

Coopers' (1950) study on normal spermatogenesis was at the light microscope level and was therefore incomplete in the terminal differentiative events. The first ultrastructural study of spermiogenesis in Drosophila was limited to the Nebenkern derivatives and axoneme substructure in wild-type flies (Yasuzumi, Fujimura and Ishida, 1958). Meyer (1964), studying the paracrystalline structure of the primary Nebenkern derivative, presented evidence indicating that respiratory enzymes are stored in the paracrystalline structure. He also postulated a supportive role for the paracrystalline structure in the mature sperm tail. Shoup (1967) studied morphogenesis of normal sperm nuclei and aberrant nuclear change in a X-chromosome male-sterile stock. She found a lack of nuclear elongation and, at the histochemical level, loss of the normal conversion of lysine-rich to arginine-rich histone in the mutant nuclei.

Anderson (1967), studying the effect of temperature on normal sperm differentiation, ultrastructurally characterized normal spermiogenesis at 18°C and 25°C and found that the temperature has no significant effect on organelle structure and development. Perotti (1969) published a study on the structure of the mature spermatozoan of Drosophila but did not discuss the spermatogenetic process to any length. Tates (1971) presented the most complete study on normal spermatogenesis at the fine structure level. His study treated the premeiotic and meiotic differentiative stages in a

comprehensive fashion, but was notably deficient in the subtle description of later spermatid differentiation. Stanley, et al. (1972) have prepared a comprehensive study of organelle relationships and spermatid differentiation that provides a reference of wild type spermatogenesis against which the experimental studies presented in this thesis are compared.

Genetic control of spermiogenesis in Drosophila

Most of the work on spermiogenesis has been restricted to deletions and mutations of the Y chromosome. Safir (1920) showed that X/0 males produced no motile sperm but were otherwise normal. Brosseau (1960) studied mutations and deficiencies of the Y chromosome finding normal premeiotic stages but failure of the spermatids to elongate and mature normally.

Meyer, Hess and Beerman (1961), Hess and Meyer (1968) and Meyer (1969) studied the ultrastructural effects of Y-chromosome mutations and deletions on spermiogenesis. Although some variation was noted between species, they concluded that Y-chromosome gene products serve an organizational rather than a structural role in Drosophila melanogaster. Kiefer (1966, 1968, 1969 and 1970) has studied spermiogenesis in Y-chromosome mutants and deletions of Drosophila melanogaster at the ultrastructural level and also found that there was a lack of organization of spermatid components rather than the absence of some structural entity. In the mutants he studied the ultrastructural abnormalities were limited to the Nebenkern derivatives and the axonemal complex. Bairati and Baccetti (1966) found in studying $X^{CL}Y^{S}$ male-sterile mutants, that the Nebenkern derivative formation and separation of the sperm from the syncytial mass were aberrant.

The above conclusions concerning the function of the Y chromosome on spermatid organization during differentiation suggest that chromosomes other

than the Y function in sperm differentiation. Shoup (1967), as described above, found that in a male-sterile mutant stock that also had a translocation between the X chromosome and chromosome two, nuclear elongation and the normal conversion of lysine-rich to arginine-rich histone were absent.

With ultraviolet radiation, Edmonton (see data in Lindsley and Grell, 1967) induced several male-sterile mutants on chromosome two, but the stocks were discarded before studies such as those described here could be initiated. Romrell, Stanley and Bowman (1972a, b) have isolated and ultrastructurally characterized spermiogenesis in two male-sterile mutants of chromosome two. Their data indicate autosomal gene products have a specific function in maintenance of sperm integrity and reproductive capability. The data also suggests that these genes function in the organization and direction of spermiogenesis in Drosophila.

MATERIALS AND METHODS

The recessive male sterile mutants of the third chromosome, ms(3)10R and ms(3)3R, used in this study were recovered from a Canton-S wild type stock following treatment with ethyl methanesulfonate (EMS) (Romrell, et al., 1972a, b). Following isolation, the stock was balanced with a multiple inversion chromosome that allowed only homozygous male sterile and heterozygous flies to survive (Romrell, et al., 1972a, b). The homozygous female flies in both mutants were also sterile. All flies were maintained at 25±1°C on standard corn meal-sucrose-yeast-agar medium with propionic acid added as a mole inhibitor.

In order to determine sterility of the mutant flies used for fine structural analysis, white virgin females were crossed to presumed male sterile flies. This procedure provided controls on both male sterility and on female virginity.

Light microscopic studies were made with a Zeiss WL phase microscope. Testes were dissected in Drosophila Ringers solution (Butterworth, Bodenstein and King, 1965) and either squashed under a coverslip and directly observed or stained with acetocarmine-fast green (Dippell, 1955) prior to observation. Males were examined for testis shape, ducts, accessory glands and ejaculatory bulb as well as for sperm motility. The spermathecae and seminal receptacles of females used in sterility checks were observed for the presence of sperm.

For electron microscopy, testes from sterile males were dissected in 2.5% glutaraldehyde, fixed for one hour in the same solution and then post-fixed for one hour in 1% osmium tetroxide. Both fixatives were in 0.2M sodium cacodylate at pH 7.4. Better preservation of membranes was noted in mutant ms(3)10R by the substitution of 0.1M sodium cacodylate at pH 7.4 in

the above fixatives, or by the use of the simultaneous fixation described by Stanley (1971). This consists of 1.25% glutaraldehyde and 1.25% osmium tetroxide in 0.1M sodium cacodylate at pH 7.4. Following fixation, the testes were rinsed in buffer, dehydrated in a graded series of ethyl alcohol solutions, cleared in three changes of propylene oxide and embedded in Epon 812 (Luft, 1961). Sections were cut with either a diamond or glass knife on a Sorvall MT-II ultramicrotome and were picked up on carbon coated grids. The sections were then doubly stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). For negative staining, testes were teased apart in distilled water, picked up on carbon coated grids and then stained with 1% sodium phosphotungstate at pH 7.2. All electron microscopic observations were made with a Zeiss EM-9S-2 electron microscope.

RESULTS

I. Autosomal male-sterile mutant ms(3)10R

A. Light microscopy.

With the exception of the seminal vesicles, the male reproductive system of ms(3)10R is complete and appears to be normal. The seminal vesicles, which normally have a diameter approximately one and one half times that of the testis, are reduced to a diameter approximately equal to that of the testis itself. Late spermatid nuclei are occasionally observed with the aid of fast green staining to be out of register with respect to each other compared to wild type spermatids whose heads are in close lateral alignment.

No motile sperm were seen in mutant flies. Both light and electron microscopic observation showed that transfer of sperm to the female reproductive system did not occur, although copulation was observed with confirmed male sterile flies.

B. Electron microscopy of spermatogenesis.

In normal spermatogenesis in Drosophila melanogaster, predefinitive spermatogonia divide to give definitive spermatogonia. The latter then divide synchronously to give 16 primary spermatocytes which subsequently undergo meiosis (Cooper, 1950). Normally the spermatids undergo differentiation that results in spermatozoa with two Nebenkern derivatives, a single axoneme, a narrow elongate head and a lanceolate acrosome (Stanley, et al., 1972; Tates, 1971).

The apical and spermatogonial cells found in the germinal tip of the ms(3)10R testes are consistent in morphology with the description given by Tates (1971). The apolar spermatocytes appear morphologically normal with the exception of the nucleolus. In the mutant cells the nucleoli appear

more distinctly fibrous, containing many strands about 165 Å in diameter (figures 1 and 2). Although such fibers are occasionally observed in wild type nucleoli, they are more distinctive in the nucleoli of this mutant. It appears that the greater visibility of the coarse fibers may be due to dispersion of granular material that normally surrounds the fibers (figure 2).

Large myelin figures are consistently found in the meiotic division stages of this mutant (figure 3). The spindle microtubules and the centrioles appear to be normal.

The acrosome in the spermatids of this mutant form from an extensive Golgi complex as in wild type. In the late stages of development, the acrosome elongates and exhibits projections that extend to the plasma membrane (figures 7 and 8). These projections are also found in wild type spermatids, except that the wild type acrosome is also surrounded by a population of cytoplasmic microtubules. Sometimes the acrosome appears somewhat contorted (figure 4) but otherwise normal.

In stage 7 wild type spermatids, the nuclei show distinct alignment of the chromatin along the inner surface of the nuclear envelope. Such alignment of chromatin appears opposite cross-linked cytoplasmic microtubules that extend around most of the periphery of the nucleus (Stanley, et al., 1972). In the majority of the stage 7 mutant nuclei, condensed chromatin is concentrated along the inner side of the nuclear envelope in a thick and apparently randomly oriented layer (figure 8). Such nuclei do not elongate and no closely associated microtubules are observed over the outer nuclear surface. A large central area of medium electron opacity is characteristic of these nuclei. A few more-normal-appearing nuclei are also seen in mutant testes. These have a microtubular layer associated with the outer nuclear surface, but component tubules are less numerous than in normal spermatids

(figures 9 and 10). Chromatin condensation at the inner nuclear surface appears to be essentially similar to that in wild type. Some areas lacking microtubules also show chromatin peripheralization. In a single instance a nuclear cross section was observed that appeared to have undergone nearly normal condensation (figure 15). An essentially complete microtubular array was observed around this nuclear section. In confirmation of observations on isolated bundles by light microscopy, sections of intact testes showed a scattered spatial distribution of spermatid nuclei within a bundle.

Counts of sperm bundles at any developmental stage in wild type regularly show 64 axonemes and 64 each of primary and secondary Nebenkern derivatives. Counts made on ms(3)10R mutant showed up to 91 discernable axonemal cross sections and as many as 185 discernable paracrystalline structures per bundle. Within bundles exhibiting greater than the normal number of sperm, there were axonemes whose doublet orientations were reversed (figures 9 and 14) indicating the possibility that some axonemal sections represent recurved portions of other axonemal images seen in the same area. Identifiable in these bundles are numerous dissociated axonemal components linked in normal fashion with smooth endoplasmic reticulum (figures 6 and 13). Occasionally, two sets of central doublets are seen in the axoneme (figures 5 and 12). This configuration has only been observed in axonemes that are at least partially disrupted.

Also in these aberrant bundles there are long, tortuous Nebenkern derivatives in which are found as many as four images of paracrystalline bodies, but most generally only one or two (figures 8 and 11). The paracrystalline structure in the Nebenkern derivative is almost always found adjacent to an area where the derivative contacts a section of smooth endoplasmic reticulum. Negatively stained paracrystalline bodies in mutant

spermatids demonstrate that they have a periodicity within the range determined for wild type (about 290 Å major longitudinal periods). The paracrystalline structure apparently begins to degenerate in stage 10 spermatids (figure 16).

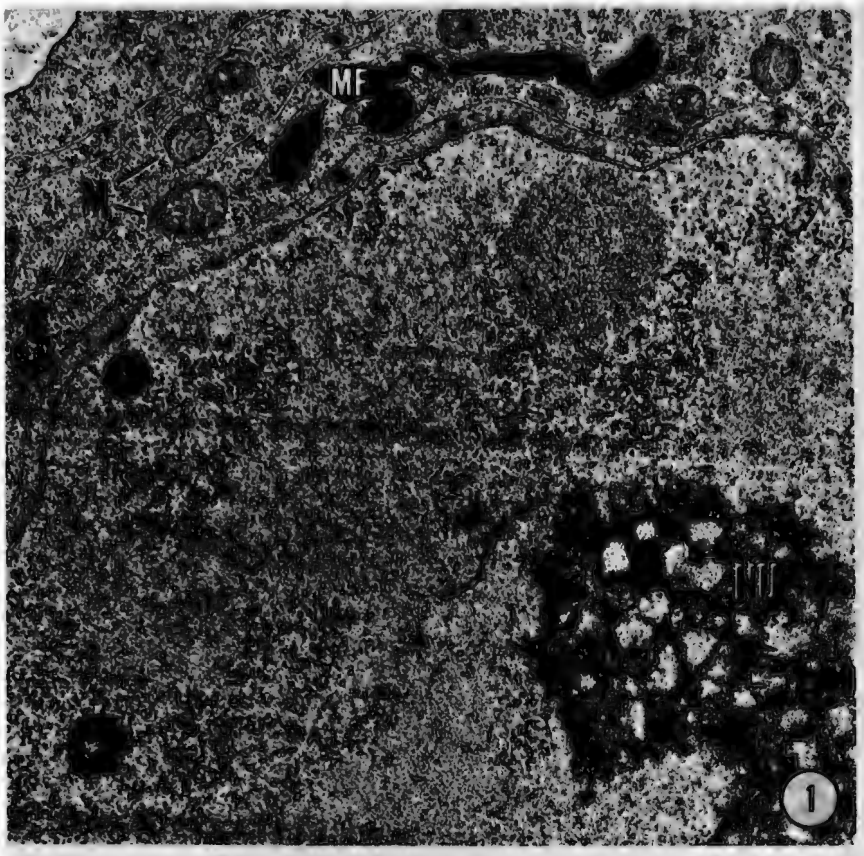
Based on the extent of axonemal development, mutant spermatids differentiate to a point equivalent to stage 10 (Stanley, et al., 1972). By this stage cytoplasmic aggregations of electron dense coarse granules are seen. This increased cytoplasmic density is accompanied by an increased distance between cytoplasmic masses that contain the spermatids (figure 17).

In the seminal vesicles, spermatids degenerate further by the breaking up of membranes and dispersion of cytoplasmic elements. Scattered paracrystalline bodies lacking complete membranes are found interspersed with cored microtubules and assorted membranous and granular fragments (figures 18 and 19). No intact sperm were observed in the seminal vesicles.

Figure 1. Section of mutant testis showing an apolar spermatocyte. The mitochondria (M) and myelin figures (MF) in the cytoplasm appear normal. The nucleolus (NU), however, has a fibrous appearance. Other nucleic inclusions are normal. 19,000X.

Figure 2. Higher magnification of the nucleolus seen in figure one. The fibers (arrows) are about 165 Å in diameter. 45,500X.

Figure 3. Section through a dividing primary spermatocyte (first meiotic division) showing spindle microtubules (MT) and large myelin figures (MF). The centrioles (C) appear normal in this stage. 45,500X.


MF
MT
1


2


MT
3

Figure 4. Nucleus (N) of an early spermatid that has failed to elongate. The acrosome (A) appears aberrant possibly because of the lack of nuclear elongation. 45,500X.

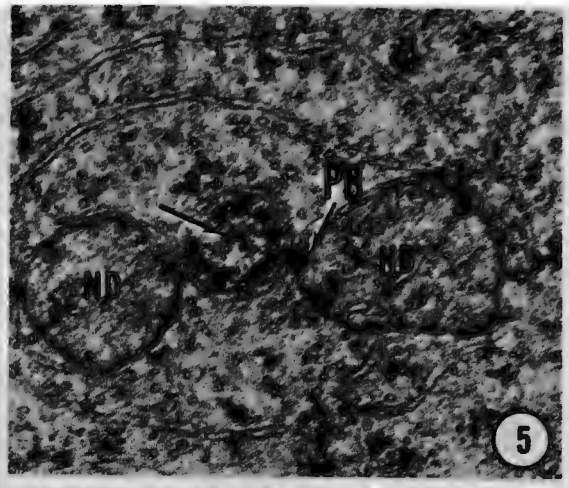
Figure 5. Axoneme of an early spermatid. The paracrystalline body (PB) has just started to form in one of the Nebenkern derivatives (ND). This axoneme has two sets of central doublets (arrow) and is disrupted. 44,000X.

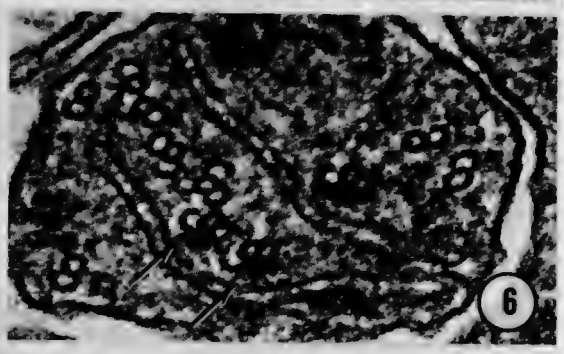
Figure 6. Doublets of an early axoneme that shows their association with smooth endoplasmic reticulum (arrows), regardless of their disruption from the axoneme. 123,000X.

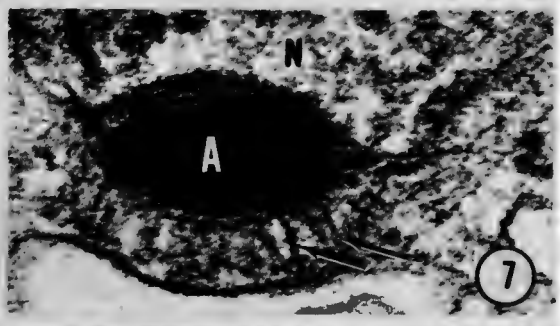
Figure 7. Normal appearing acrosome (A) showing the links that exist from the acrosome to the plasma membrane (arrows). A portion of the nucleus (N) is seen in this section. 132,000X.

Figure 8. Nucleus of a stage seven spermatid showing abnormal chromatin (CH) condensation that leaves an area of reduced electron opacity in the center of the nucleus (NV). The attached acrosome (A) has the projections that normally exist to the plasma membrane. Several abnormal appearing Nebenkern derivatives (ND) are seen (arrows). 37,500X.


A
4


PB
ND
ND
5


6


N
A
7

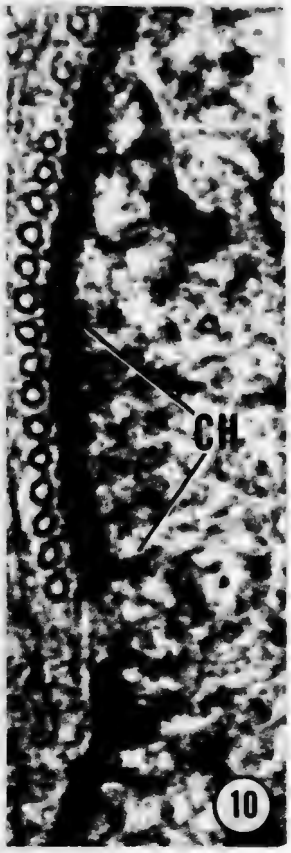
A
KV
CH
8

Figure 9. Section through a sperm bundle in stage seven. Compare the nucleus (N) seen in this section with that of figure 8. Note the reverse orientations of the doublets in the axonemes indicated (arrows). A portion of a nurse cell (NC) is seen. 37,500X.

Figure 10. Higher magnification of a portion of the nucleus seen in figure 9. Note the incomplete microtubular array and the pattern of chromatin condensation (CH). 123,000X.

Figure 11. Section through the flagellar region of a stage seven sperm bundle. Note the sinuous Nebenkern derivatives (ND) and their loss of attachment to the periaxoneal endoplasmic reticulum. Axonemes are seen in several disrupted configurations. 40,000X.


NC
9


CH
10

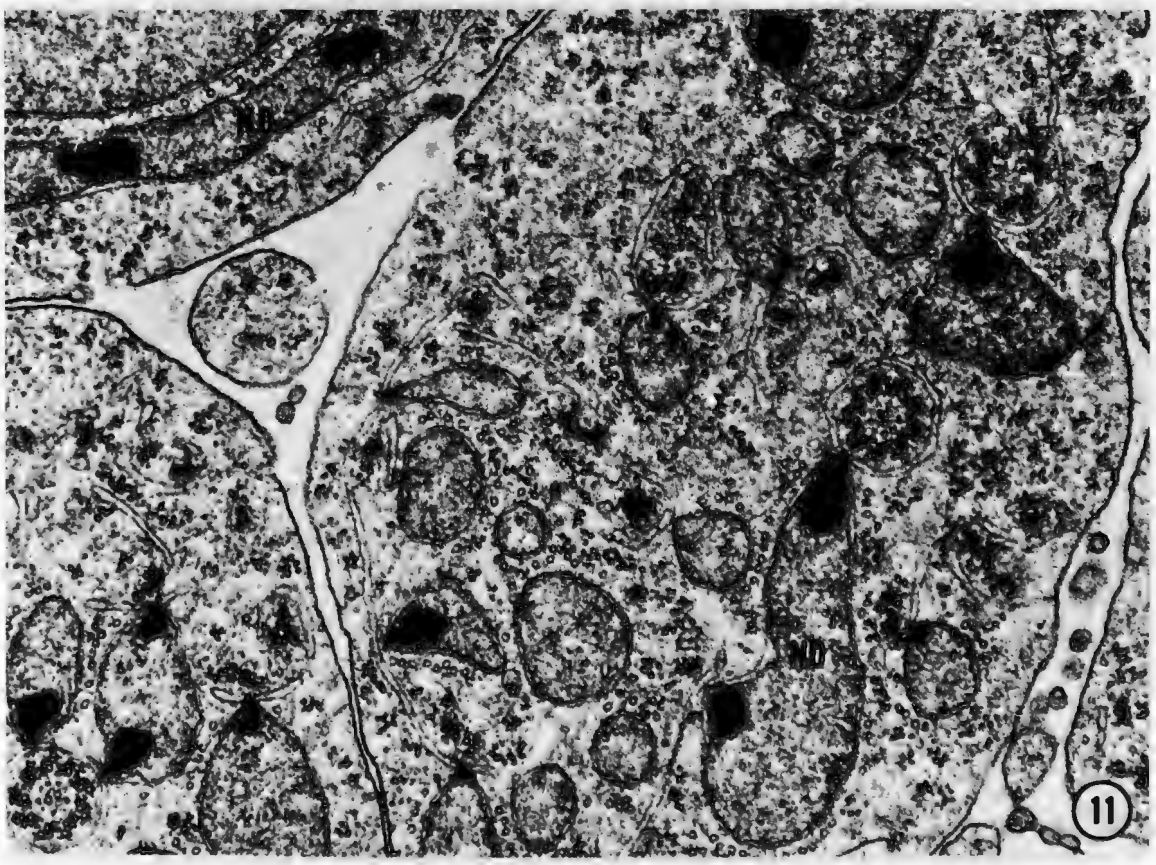

MI
11

Figure 12. A disrupted flagellar complex in stage nine. This axoneme has two sets of central doublet tubules (CT) and lacks one set of peripheral doublets (arrow). There is a single paracrystalline body (PB) in the prominent Nebenkern derivative. 106,500X.

Figure 13. Portion of a disrupted axoneme showing the normal association between smooth endoplasmic reticulum and the accessory tubules (arrows). 148,500X.

Figure 14. Stage ten sperm tails demonstrating the reverse orientation of the axonemes within a common cytoplasmic matrix. Compare the doublet orientation of the two axonemes indicated (arrows). 46,500X.

Figure 15. The only late nucleus (stage 10) found having a morphologically normal appearance. The microtubular array (although oblique in plane of section) is nearly complete. 123,000X

Figure 16. Late sperm tail showing the apparent degeneration of the Nebenkern derivative by swelling of the membrane complex away from the paracrystalline structure (PB). There is a change in the morphology of the paracrystalline body at its periphery (arrows) possibly signalling its degeneration. 98,000X.

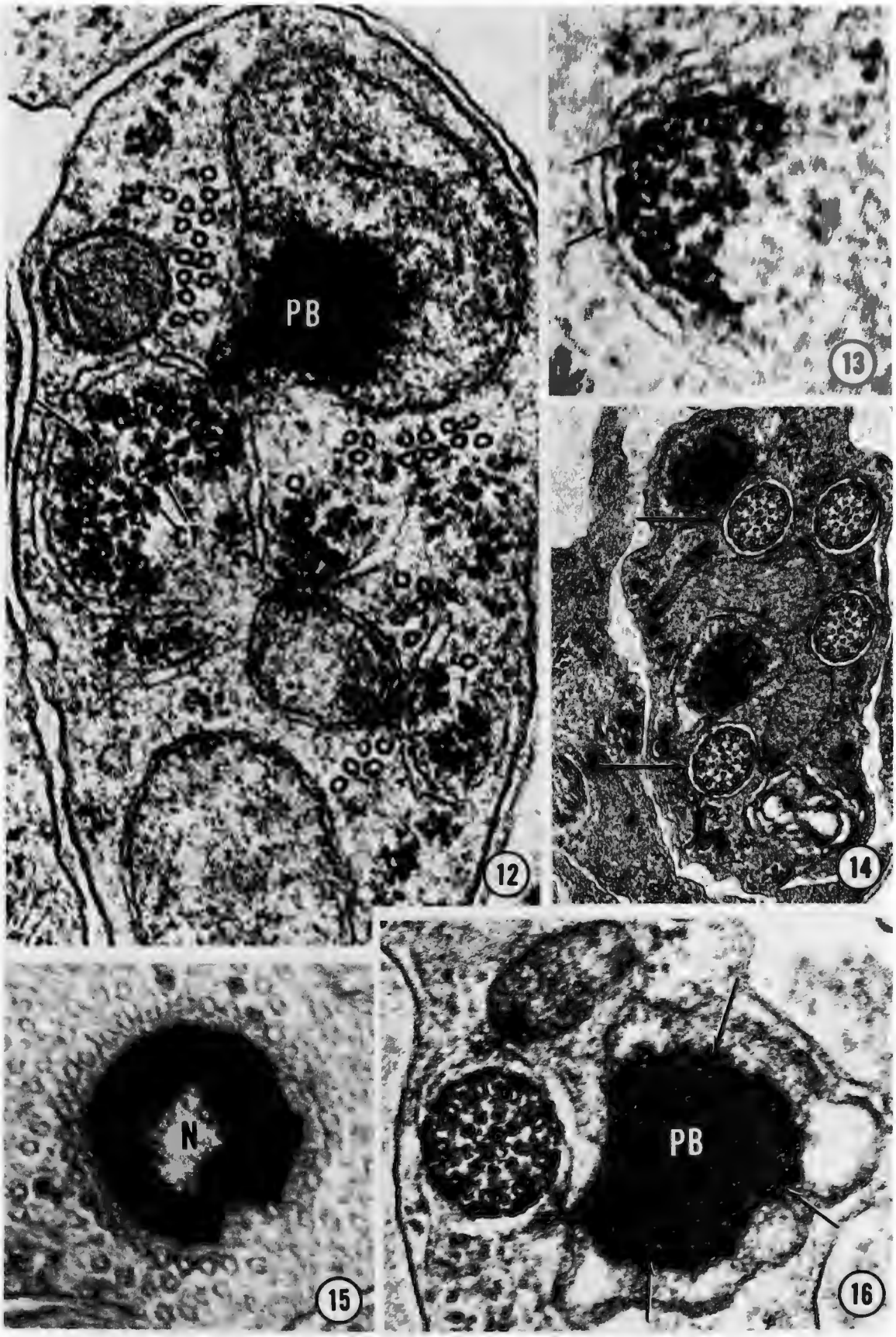
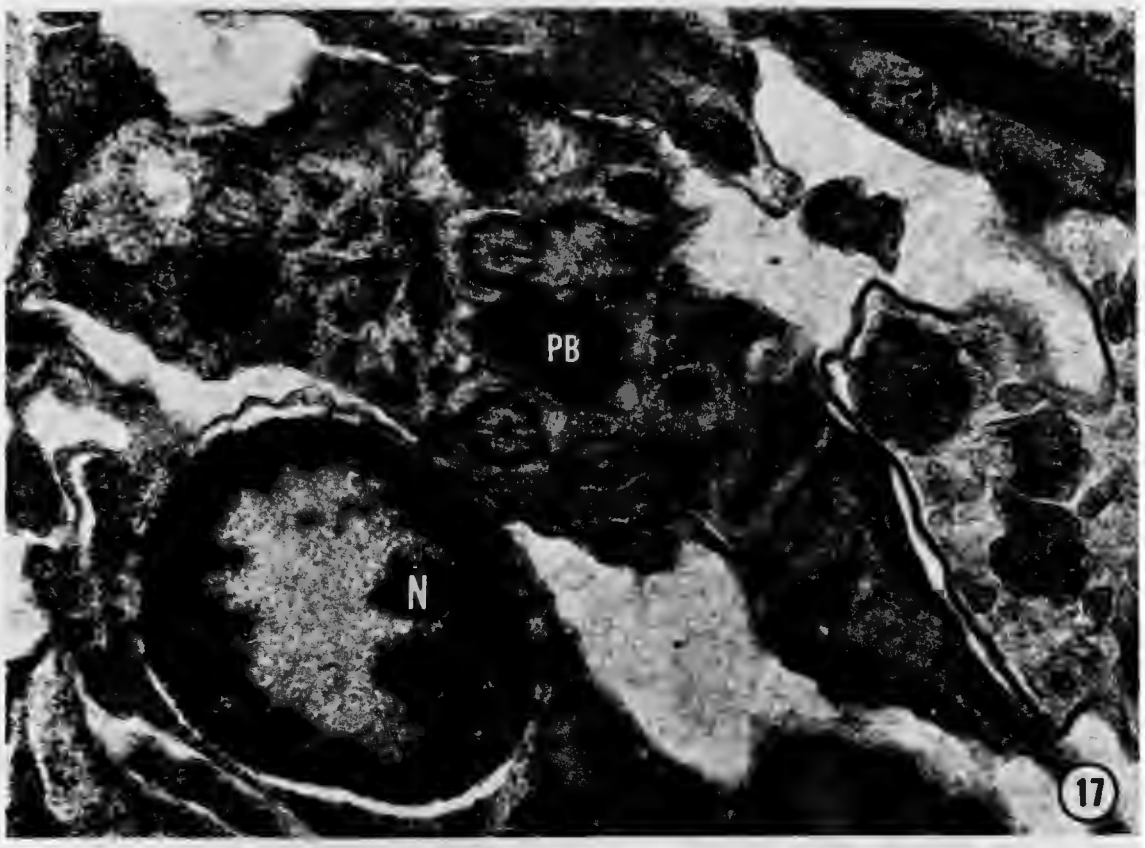
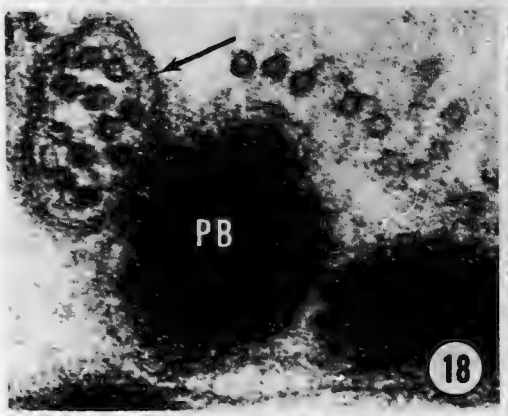

PB
12
13
14
N
15
PB
16

Figure 17. Late stage of spermiogenesis showing a bundle in degeneration. The nucleus (N) has failed to elongate but shows peripheralization of the chromatin. There is an increase in the overall electron density and an increase in the granular nature of the cytoplasm surrounding the sperm. There are also increased distances between the cytoplasmic matrices that contain the sperm (*). 38,400X.

Figure 18. Cored axonemal microtubules in the seminal vesicle in association with paracrystalline structures (PB). Very little membranous structures are present (arrow). 134,500X.

Figure 19. Survey micrograph of the seminal vesicle showing disrupted and scattered microtubules (MT). Paracrystalline structures (PB) are seen in oblique section. 29,500X.


PB
N
17


PB
18

MT
PB
19

II. Autosomal male-sterile mutant ms(3)3R

A. Light Microscopy.

The reproductive system of ms(3)3R is normal with the exception of the seminal vesicles, which are reduced to a diameter equal to that of the testis. (Wild type seminal vesicles are generally about one and one half times the diameter of the testis.) The males of this mutant do not copulate and, of course, there was no transfer of sperm to the female reproductive system in any of the flies used in the sterility checks.

B. Electron microscopy of spermiogenesis.

In normal spermiogenesis there are two Nebenkern derivatives of about equal size that arise from the division of a mitochondrial syncytium termed the Nebenkern. Of these two derivatives, one forms a paracrystalline inclusion and the other becomes much reduced as viewed in cross sections of late spermatids. The nucleus characteristically undergoes elongation associated with condensation of the nuclear contents. The acrosome originates from an extensive Golgi complex and forms a lanceolate structure on the apical end of the nucleus. The axoneme originates from a centriole and a centriolar adjunct at the base of the maturing nucleus. The mature axoneme shows a typical inset "9+9+2" microtubule pattern in cross section. For a detailed description of spermiogenesis in Canton-S wild type, see Stanley, et al., 1972.

Abnormalities at the ultrastructural level in ms(3)3R, which appear to be limited, at least initially, to the sperm tail, begin at a stage corresponding to stage 10 in Canton-S wild type (Stanley, et al., 1972)(figure 20). The primary derivative in most instances loses its direct association with the periaxonemal endoplasmic reticulum. Relative to wild type there is a marked variation in the cross sectional area of the secondary derivative and

in the retention of cytoplasmic matrix around the developing sperm. Subsequently, the sperm in a degenerating bundle coalesce, a change accompanied by the loss of large quantities of membranous and granular material (figure 21). The location of the lost material, apparently the cytoplasm and surrounding membrane of the sperm, in the nurse cell cytoplasm suggests a phagocytic role of the nurse cell in association with a developing sperm bundle. There is loss of discernable secondary derivatives in the coalesced bundles, and it appears that the primary derivatives begin to degenerate as evidenced by the loss of the close association of the paracrystalline body with the inner membrane of the mitochondrial derivatives (figure 22). The axonemes are intact and have developed such that there is no morphological or dimensional deviation from the wild type axoneme.

The nuclei and acrosomes at this stage appear normal although there is apparently a retention of cytoplasmic matrix in this area (figure 23). The coalescence does not extend to the nuclear region.

As the degenerating sperm bundles enter the seminal vesicles, the axonemes frequently appear collapsed, apparently due to the loss of the 'A' subtubule and its' associated spoke (figure 24). The paracrystalline structure of the primary Nebenkern derivative is maintained, but there are multilaminar structures among the disrupted axonemes and paracrystalline bodies. The 'B' subtubule of each peripheral doublet is subsequently lost leaving 11 cored tubules, the two central and nine peripheral accessory tubules of the axoneme (figure 25). The central tubules are identified by the link that persists between them, and the peripheral accessory tubules are identified by their laterally projecting arms. A further stage in degeneration involves the loss of the electron dense coating around the cored tubules leaving a thin-walled cored cylinder or tubule (figure 26). In all

the stages described above, the tubular components are intermixed with paracrystalline bodies and multiple layers of membranes. It is assumed that the membrane configurations originate from the preexisting derivative, axonemal, and plasma membranes of the spermatozoa. Further degeneration results in the loss of arms or cross-links associated with the tubules (figure 27).

In addition to the sequence of degenerative changes described above, complete, but often disrupted axonemes are found in the seminal vesicles (figures 28 and 29). In contrast to the degenerative sequence previously described, the disrupted axonemes maintain the integrity of the doublet, spoke and accessory tubule complex (figure 29). Occasionally, axonemes are found that have lost some of the electron dense material associated with the accessory tubules (figure 30). With these configurations there is a marked absence of the laminar membrane arrays characteristic of the sequence of degenerative events described above. Degenerative changes that follow probably occur in the absence of such laminar arrays and, therefore, degenerating sperm are scattered randomly throughout the seminal vesicles, making it impossible to determine an ordered sequence of degeneration.

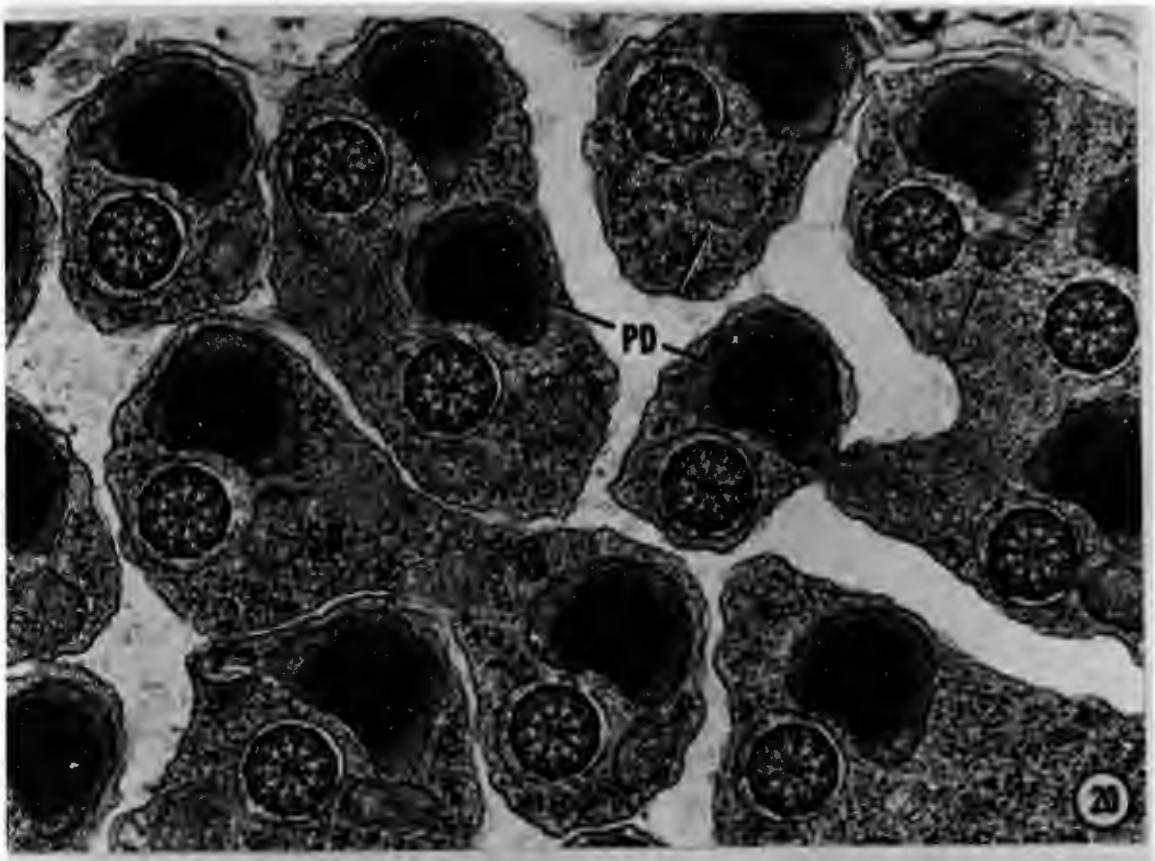
Nuclei of sperm in the seminal vesicle appear to be normal. There is no evidence of retention of cytoplasmic matrix as described for the late testicular nuclei.

Since ms(3)3R mutant males do not copulate, the degenerative changes noted could possibly occur in wild type males. To test this, Canton-S males were recovered two to three hours after eclosion and were aged in the absence of females for 15 days. The testes were then observed at the light and electron microscope levels. The seminal vesicles were greatly enlarged, reaching a diameter approximately two and one half times that of the testis.

Sections through these seminal vesicles showed no distinct ultrastructural changes other than some swelling of spermatozoa resulting in a separation of the outer membrane complex from the axoneme and paracrystalline body (figures 31 and 32).

Figure 20. Section through a mutant sperm bundle in Stage 10 of development (Stanley, et al., 1972). There is retention of a large amount of cytoplasm around the individual spermatids. Primary Nebenkern derivatives (PD) and secondary Nebenkern derivatives (SD) are shown. Note also the relatively large variation in cross sectional area of the secondary derivatives (arrows). 45,000X.

Figure 21. Portion of a coalescing sperm bundle (SB) showing membranous material (MM) and granular material (GM) located in the nurse cell (NC) cytoplasm. 13,000X.


PD
20

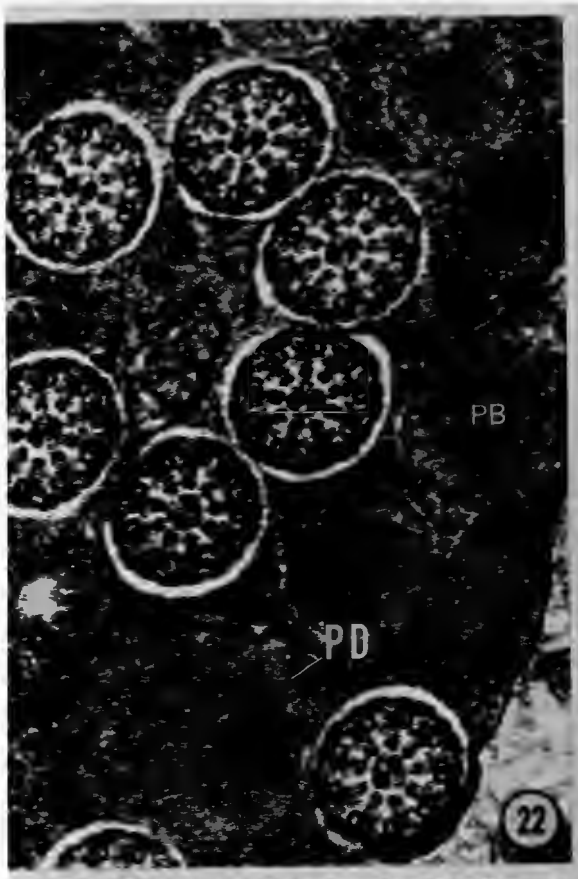

SB
MM
NC
MM
GM
21

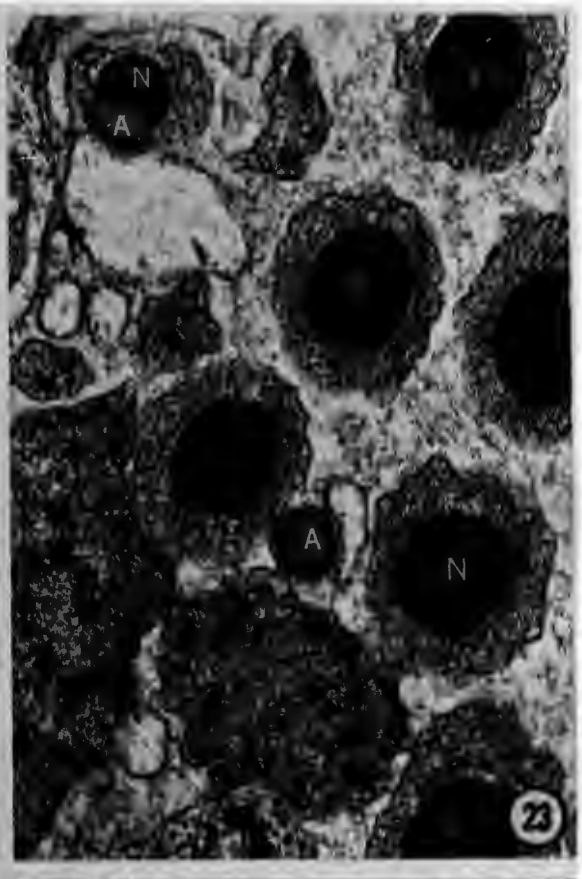
Figure 22. Early stage of degeneration within a coalesced sperm bundle showing the absence of secondary Nebenkern derivatives and the loss of close association between the paracrystalline body (PB) and the membrane complex of the primary Nebenkern derivative (PD). 78,500X.

Figure 23. Section through a sperm bundle in the nuclear region. Although bundle coalescence does not extend into the nuclear region, there is a large amount of cytoplasm around the nuclei (N). Acrosomes (A) are also present in this section. 45,000X.

Figure 24. A section demonstrating a later stage in axonemal degeneration showing loss of the inner portion of the 'A' subtubule and associated spoke (arrow). Multilaminar structures (ML) have formed around the paracrystalline body (PB) and the axonemal remnant. 161,000X.

Figure 25. Micrograph illustrating the stage at which there is loss of all doublet structures leaving only the cored central (CT) and accessory (AT) microtubules in close juxtaposition. Multilaminar structures (ML) surround both the cored axoneme microtubules and the paracrystalline body (PB). 228,000X.


PB
PD
22


N
A
A
N
23


PB
ML
24

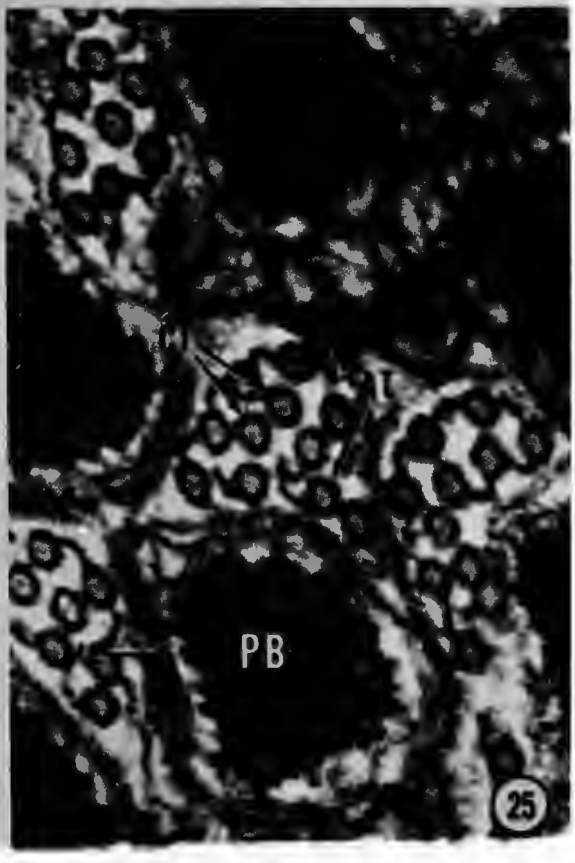

PB
25

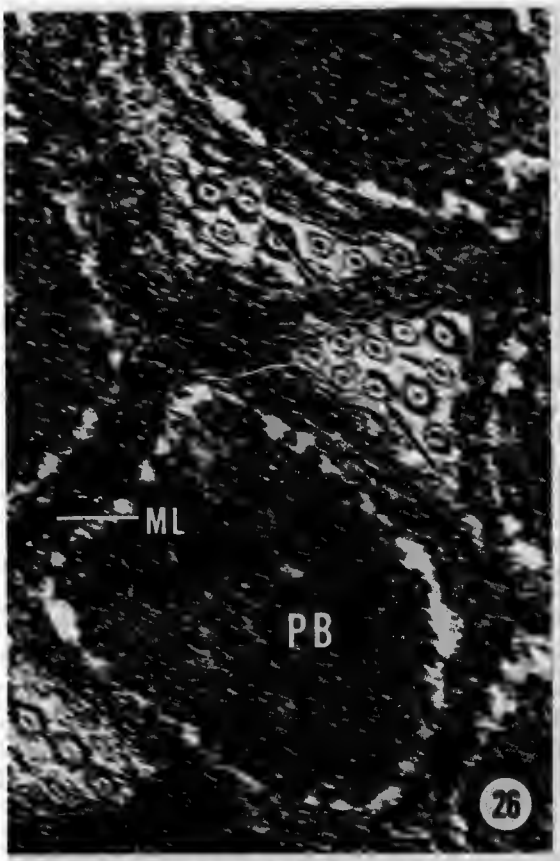
Figure 26. Stage of axoneme degeneration showing loss of the electron dense coating around the cored central and accessory microtubules (arrows). The multilaminar structures (ML) around the axoneme and paracrystalline body (PB) reach their greatest thickness in this stage. 244,000X.

Figure 27. Stage at which only the denuded axonemal central and accessory cored microtubules remain. The multilaminar structures (ML) and paracrystalline bodies (PB) appear to be greatly diminished in size. 244,000X.

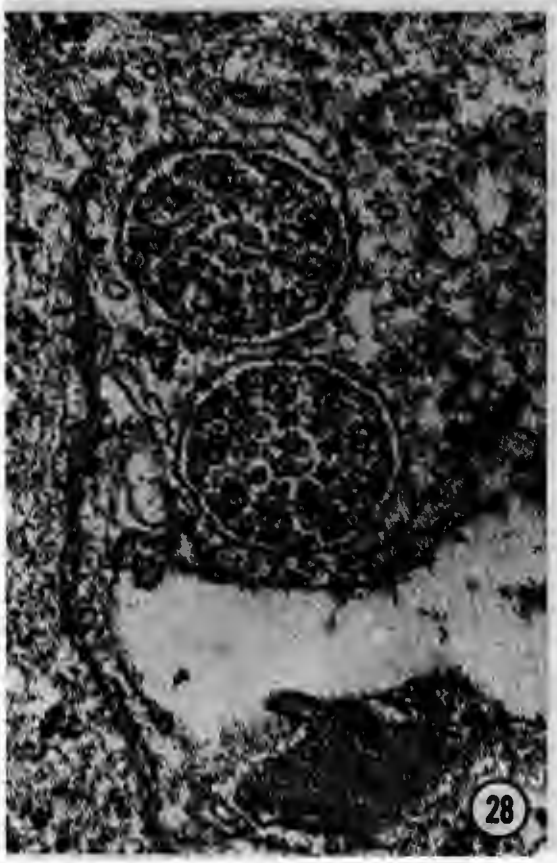
Figure 28. Morphologically normal appearing axonemes found in the seminal vesicles. They have lost their association with primary or secondary Nebenkern derivatives. 98,000X.

Figure 29. Section of seminal vesicle in an area where axonemes have not started to degenerate. Disrupted axonemes show maintenance of the accessory tubule, peripheral doublet and spoke structure (arrows). Paracrystalline bodies (PB) are present but the multilaminar structures characteristic of degeneration in the coalesced bundles are absent. 70,000X.

Figure 30. Detail of a complete axoneme from the seminal vesicle. Note the loss of the electron dense material associated with the accessory tubule (arrows). 228,000X.


ML
PB
26


PB
27


28

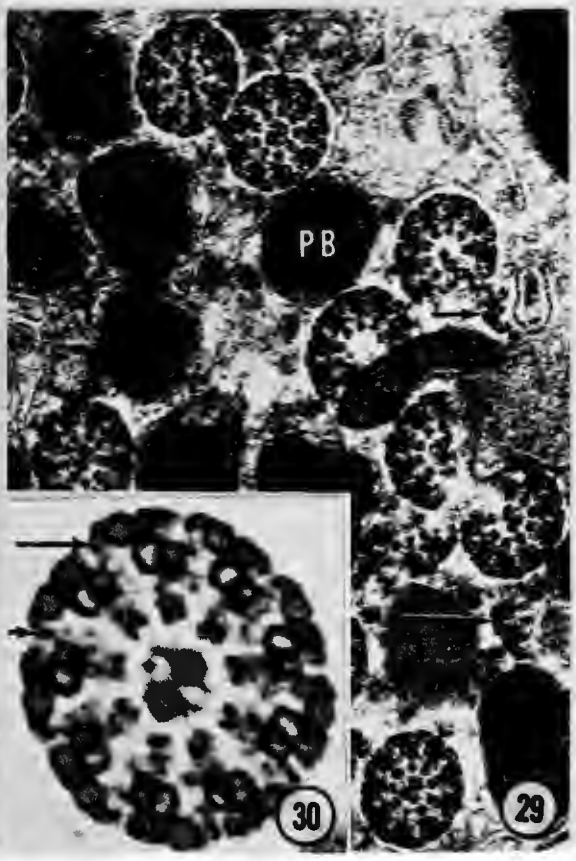
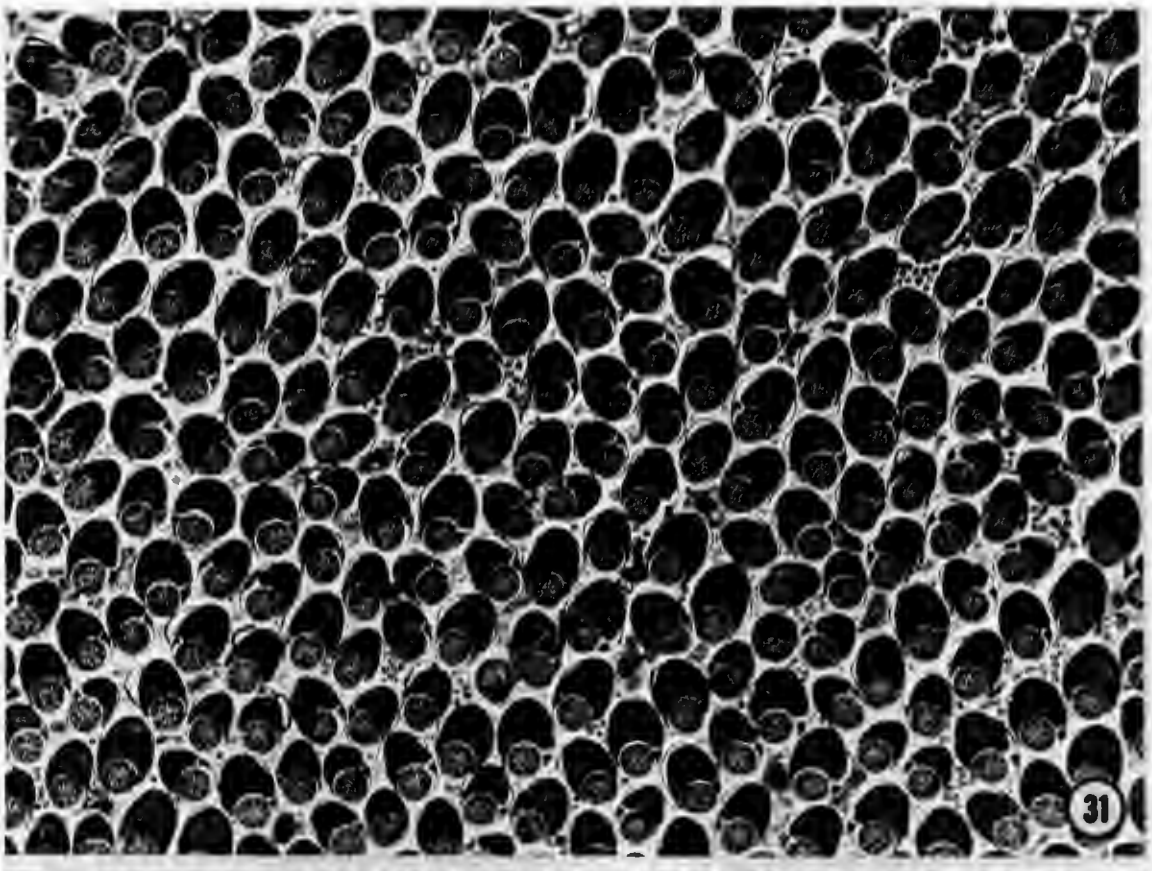

PB
30
29

Figure 31. Section through a Canton-S wild type seminal vesicle after aging in the absence of females for 15 days after eclosure. The subtle variation in the cross sectional area of the primary Nebenkern derivative suggests that the sperm tail is tapered. A nucleus (N) is present in this section. 18,000X.

Figure 32. Detail micrograph of sperm tails in a seminal vesicle of an aged male as described in the legend for figure 12. The axoneme, primary derivative (PD) and secondary derivative (SD) appear normal. The membrane complex around the sperm tail is swollen away from the surface of the axoneme and derivatives (arrows). The areas of reduced electron density (RD) are seen also in primary derivatives of males aged in the presence of females. 137,000X.


31


32

DISCUSSION

I. Autosomal male-sterile mutant ms(3)10R

The major abnormalities observed in spermatids of mutant ms(3)10R are aberrant chromatin condensation and failure of nuclear elongation. Correlated with these abnormalities is a lack of perinuclear microtubule assembly such as is found in wild type males. An area of current controversy involves the possible role of cytoplasmic microtubules and/or chromatin in elongation and morphogenesis of the sperm head (Kessel, 1969; Fawcett, Anderson and Phillips, 1971). There is strong circumstantial evidence implicating extrinsic factors in the formation of the sperm head (Kessel, 1966, 1969; McIntosh and Porter, 1967). Microtubules have been implicated as the extrinsic factor involved in shaping of the head because of their often close apposition to the nuclear envelope and their disappearance once nuclear differentiation is complete. This hypothesis of microtubular involvement as a direct influence in the shaping of the sperm head has recently been confounded, however, by evidence reviewed by Fawcett, et al. (1971). The latter authors conclude that factors within the nucleus determine the pattern of chromatin condensation and this in turn is the major element in determination of ultimate head shape.

Stanley, et al. (1972) have shown that in wild type Drosophila a single layer of cross-linked microtubules is closely appressed to the nuclear surface, especially during later stages in spermiogenesis. The microtubular array is matched across the two adherent leaflets of the nuclear envelope by longitudinal ridges of chromatin. These authors postulate a causal relationship across the nuclear envelope that results in close register of elements on either side. Specifically, the microtubules may induce a specific pattern

of peripheral chromatin condensation which, in turn, may direct the elongation and shaping of the sperm head. However, it is not possible to determine with certainty the direction of the causal stimulus. The relationships described in the majority of nuclei in mutant ms(3)10R support the hypothesis of a transmembrane relationship. Nuclei lacking a peripheral array of microtubules exhibit quite abnormal chromatin pepherelization and fail to elongate. A small number of nuclei were observed that possessed partial microtubule assemblies and these areas corresponded exactly with areas of normal nuclear configuration. Only slight abnormalities of nuclear elongation were noted in such cells. Finally, a single nucleus of apparently normal aspect with regard to microtubule array and chromatin condensation was observed. Within this single mutant stock, therefore, a range of nuclei from normal to highly aberrant were observed and a constant correlation between microtubule presence and form of chromatin condensation was apparent. Since the perinuclear microtubular array normally exists before the characteristic chromatin condensation appears, it seems most likely that causal stimuli proceed from the microtubule area across the nuclear envelope to the peripheral chromatin. It is also quite possible that the specific alignment of perinuclear microtubules and peripheral chromatin are both affected by a third element such as the nuclear envelope itself.

Other morphological abnormalities noted include probable bending of axonemes back upon themselves, axonemal disruption, and aberrant shaping of the Nebenkern derivatives. All of these abnormalities may be explained as concomitants of incomplete cellular elongation. Since morphological connections among the component organelles in the mutant sperm tails appear to be less constant, it seems that the attachment of the axoneme and the Nebenkern derivatives to the axonemal sheath as seen in wild type may play

an important role in the extensive elongation of the cell as a whole.

Since the spermatids do not synthesize new RNA (Hennig, 1967, 1968) and since spermatocytes are the last cells in spermatogenesis to synthesize new RNA in Drosophila (Oliveri and Oliveri, 1965), the aberrant nucleoli seen in spermatocytes of this mutant may indicate malfunctions of this nuclear organelle. The specific malfunctions possible include improper synthesis of ribosomal RNA and the failure of other types of RNA to be transferred to the cytoplasm (Harris, 1970). This could explain the relatively large number of sperm abnormalities observed due to a single gene mutation.

II. Autosomal male-sterile mutant ms(3)3R

In the mutant ms(3)3R, the spermatids differentiate in a normal fashion to stage 10 (Stanley, et al., 1972). At this point, the sperm begin an ordered series of degenerative changes beginning with a breakdown of the limiting plasma membranes. This leaves the organelles of many sperm in a common cytoplasmic matrix. The 'A' subtubule and associated spoke in the axoneme are the next structures to disappear, followed by loss of subtubule 'B'. This leaves a random array of the 11 cored tubules (central and accessory). The electron dense material surrounding these remaining tubules disperses before the tubules and their core structures disappear. Following the early degenerative stages, multilaminar configurations appear among the Nebenkern derivatives and partially disrupted axonemes. Such configurations resemble layered unit membranes and may result from reassembly of previously disrupted plasma and endoplasmic reticulum membranes. This sequence of degenerative changes in the axoneme differs from the degenerative changes in X/0 and Y-chromosome mutants described by Kiefer (1966, 1968, 1969, 1970) in that the doublets degenerate in reverse sequence. Kiefer implies that the same order of disassembly results from a variety of genome defects and that the sequence may be dictated by the inherent tendency of some subunits to disassemble more readily than others regardless of the causes of the degenerative events. The degenerative sequence in ms(3)3R indicates that, on the contrary, specific sequences of axonemal disassembly may be the result of specific genomic abnormalities.

Mutant ms(3)3R is similar to numerous other mutant stocks studied by us (unpublished observations) and by Hess and Meyer (1968), Kiefer (1966, 1968, 1969, 1970), and Shoup (1967) in that differentiation of spermatids proceeds normally to a certain point and then general degenerative changes

ensue. Such mutations may be intrinsic or extrinsic to the germ cells, i.e., the mutation may affect a gene active in the primary spermatocyte whose product is necessary for differentiation at the stage in which degeneration begins, or the mutation may affect a gene transcribed only in certain somatic tissues such as the supportive cells of the testis or perhaps an endocrine tissue elsewhere in the body. The fact that behavior (copulation) in the present mutant stock is altered argues in favor of the latter possibility. Such a mutation could supress copulation as well as terminate spermiogenesis at some definitive stage. The gene product in question then, may have a dual function, one in the control of behavioral and the other in mediating a specific cytodifferentiative event. Hormonal control over spermatogenesis has been well established in mammals (see review by Steinberger, 1971) and it appears that in at least some insects there is hormonal control over sexual differentiation (Naisse, 1966a, b) and spermiogenesis (Cantacuzène and Seureau, 1970).

The observations on seminal vesicle sperm in aged wild-type males reported here supports the interpretation that the degenerative changes in late spermatids of mutant ms(3)3R are not due simply to their retention in the testicular duct and seminal vesicle as a result of the failure to copulate. Degeneration in the mutant appears to be due to lack of a necessary gene product at a specific stage of differentiation, whether that product is synthesized within the germ cells themselves or is produced by some somatic cell type.

SUMMARY

Spermatogenesis in two male-sterile third-chromosome mutants is described. Characteristic of sperm cytodifferentiation in the first, ms (3)10R, mutant is lack of nuclear elongation, various axoneme irregularities, highly tortous Nebenkern derivatives that often exhibit more than one paracrystalline inclusion and, lastly, aberrant nucleolar morphology in the nuclei of primary spermatocytes. Elongation of the nucleus can be constantly correlated with an association between microtubules and the outer surface of the nuclear envelope. This suggests a microtubule mediated induction of the chromatin of the differentiating nucleus that in turn functions in elongation and shaping of the sperm head. It appears that the abnormalities seen in the axoneme and the Nebenkern derivatives are due to incomplete or irregular sperm elongation.

The mutation to the second, ms(3)3R, mutant affects the late differentiation and maintenance of sperm ultrastructure. The sperm tails in late bundles coalesce concomitant with the loss of membrane and ribosome-like particles. The ultrastructural degeneration of the flagellar complex follows an ordered sequence different from the sequence described for Y-chromosome mutations and deletions. The axonemal elements, the cored central and accessory microtubules, are the most resistant to degeneration. Since this mutation also affects the behavior of the male, the primary effect may be modification or lack of a hormone with dual function, i.e. maintenance of sperm structure and behavior.

LITERATURE CITED

Bairati, A. Jr. and B. Baccetti. 1966. Observations on the ultrastructure of male germinal cells in the $X^{LC}Y^{S}$ mutant of Drosophila melanogaster Meig. D.I.S. 41: 152.

Brosseau, G. E. 1960. Genetic analysis of the male fertility factors on the Y chromosome of Drosophila melanogaster. Genetics 45: 257-274.

Butterworth, J. M., D. Bodenstein and R. King. 1965. Adipose tissue of Drosophila melanogaster. J. Expl. Zool. 158: 141-154.

Cantacuzène, A. and C. Seureau. 1970. Alterations de la spermiogenèse de Locusta migratoria migratorioides après implantation de corps allates et après irradiation. Z. Zellforsch. 103: 351-364.

Cooper, K. W. 1950. Normal spermatogenesis in Drosophila. In, Demerec, M. (ed.). Biology of Drosophila. Hafner Publishing Co., New York, New York.

Dippell, R. V. 1955. A temporary stain for paramecium and other ciliate protozoa. Stain Technol. 30: 69-71.

Fawcett, D. W., W. A. Anderson and D. M. Phillips. 1971. Morphogenetic factors influencing the shape of the sperm head. Dev. Biol. 26: 220-251.

Harris, H. 1970. The use of cell fusion in the analysis of gene action. Proc. Roy. Soc. B. 176: 315-317.

Hennig, W. 1967. Untersuchungun zur Struktur and Funktion des Lampbursten-Y-chromosoms in der Spermatogenese von Drosophila. Chromosoma 22: 294-357.

Hennig, W. 1968. Ribonucleic acid synthesis of the Y chromosome of Drosophila hydei. J. Mol. Biol. 38: 227-239.

Hess, O. and G. F. Meyer. 1968. Genetic activities of the Y-chromosome in Drosophila during spermatogenesis. Adv. Genetics 14: 171-223.

Kessel, R. G. 1966. The association between microtubules and nuclei during spermiogenesis in the dragonfly. J. Ultrastructure Res. 16: 293-304.

Kessel, R. G. 1969. Spermiogenesis in the dragonfly with special reference to a consideration of the mechanisms involved in the development of cellular asymmetry. In, Baccetti, B. (ed.), Comparative Spermatology. Academic Press.

Kiefer, B. I. 1966. Ultrastructural abnormalities in developing sperm of X/0 Drosophila melanogaster. Genetics 54: 1441-1452.

Kiefer, B. I. 1968. Y-mutants and spermiogenesis in Drosophila melanogaster. Genetics 60: 192 (Abstr.).

Kiefer, B. I. 1969. Phenotypic effects of Y-chromosome mutations in Drosophila melanogaster. I. Spermiogenesis and sterility in KL-1-males. Genetics 61: 157-166.

Kiefer, B. I. 1970. Development, organization and degeneration of the Drosophila sperm flagellum. J. Cell Sci. 6: 177-194.

Lindsley, D. L. and E. H. Grell. 1967. Genetic variations of Drosophila melanogaster. Carnegie Inst. Wash. Publ. No. 627.

Luft, J. H. 1961. Improvements in Epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409-414.

McIntosh, J. R. and K. R. Porter. 1967. Microtubules in the spermatids of the domestic fowl. J. Cell Biol. 35: 153-173.

Meyer, G. F., O. Hess and W. Beerman. 1961. Phasenspezifische funktionsstrukturen in spermatocytenkernen von Drosophila melanogaster und ihre abhangigkeit von Y-chromosom. Chromosoma (Berl.) 12: 676-716.

Meyer, G. F. 1964. Die Parakristallinen Korper in den Spermienschwanzen von Drosophila. Z. Zellforsch. 62: 762-784.

Meyer, G. F. 1969. Experimental studies on spermiogenesis in Drosophila. Genetics (suppl.) 61: 79-92.

Naisse, J. 1966a. Controle endocrinien de la differenciation sexulle chez l'Insecte Lampyris noctiluca (Coleoptere Malacoderme Lampyride). I.- Role androgene des testicules. Arch. de Biol. (Liege) 77: 139-201.

Naisse, J. 1966b. Controle endocrinien de la differenciation sexulle chez Lampyris noctiluca (Coleoptere Lampyride). III. Influence de hormones de la pars intercerebralis. Gen. Comp. Endocrin. 7: 105-110.

Oliveri, G. and A. Oliveri. 1965. Autoradiographic study of nucleic acid synthesis during spermatogenesis in Drosophila melanogaster. Mutation Res. 2: 366-380.

Perotti, M. E. 1969. Ultrastructure of the mature sperm of Drosophila melanogaster Meig. J. Submicr. Cytol. 1: 171-196.

Reynolds, E. 1963. The use of lead citrate at high pH as an electronopaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Romrell, L. J., H. P. Stanley and J. T. Bowman. 1972a. Genetic control of spermiogenesis in Drosophila melanogaster: an autosomal mutant (ms(2)3R) demonstrating failure of meiotic cytokinesis. J. Ultrastructure Res. 38: 563-577.

Romrell, L. J., H. P. Stanley and J. T. Bowman. 1972b. Genetic control of spermiogenesis in Drosophila melanogaster: an autosomal mutant (ms(2)10R) demonstrating disruption of the axonemal complex. J. Ultrastructure Res. 38: 578-590.

Safir, S. R. 1920. Genetic and cytological examination of the phenomena of primary non-disjunction in Drosophila melanogaster. Genetics 5: 459-487.

Shoup, J. R. 1967. Spermiogenesis in wild type and in a male sterility mutant of Drosophila melanogaster. J. Cell Biol. 32: 663-675.

Stanley, H. P. 1971. Fine structure of spermiogenesis in the elasmobranch fish Squalus suckleyi. I. Acrosome formation, nuclear elongation and differentiation of the midpiece axis. J. Ultrastructure Res. 36: 86-102.

Stanley, H. P., J. T. Bowman, L. J. Romrell, S. C. Reed and R. F. Wilkinson. 1972. Fine structure of normal spermatid differentiation in Drosophila melanogaster. J. Ultrastructure Res. Submitted for Publication.

Steinberger, E. 1971. Hormonal control of mammalian spermatogenesis. Physiol. Rev. 51: 1-22.

Tates, A. D. 1971. Cytodifferentiation during spermatogenesis in Drosophila melanogaster. Thesis. University of Leiden. Leiden, Netherlands.

Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4: 475-475.

Yasuzumi, G., W. Fujimura and H. Ishida. 1958. Spermatogenesis in animals as revealed by electron microscopy. V. Spermatid differentiation of Drosophila and grasshopper. Expl. Cell Res. 14: 268-285.

VITA

Richard F. Wilkinson

Candidate for the Degree of

Master of Science

Thesis: Spermiogenesis in two Third-Chromosome Male-Sterile Mutants of *Drosophila melanogaster*

Major Field: Zoology

Biographical Information:

Personal Data: Born in Los Angeles, California, January 25, 1944, son of Richard F. and Doris Courtion Wilkinson; married Judith Ann Polodna on September 3, 1966. Two children, Mike and Mark.

Education: Attended elementary schools in Alhambra and Monterey Park, California; graduated from Mark Keppel High School, Monterey Park, California in 1962, attended Los Angeles State College, Received an Associate of Arts Degree from Pasadena City College in 1965 and received the Bachelor of Science degree from California State Polytechnic College, Pomona, California, in 1968 with a major in Biological Science (option in microbiology).

Professional Experience: Laboratory technician in Microbiology and Health Services at Cal Poly College, 1966-1968; Assistant Microbiologist, Los Angeles County Sanitation Districts, 1967; Microbiologist, Space-General Corporation, 1968; NDEA Fellow, Utah State University, 1968-1971; NIH predoctoral trainee, Utah State University, 1971-present.